

# Effects of vitamin C treatment on collar-induced intimal thickening

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**Abstract:** Vitamin C has efficient antioxidant properties and is involved in important physiological processes such as collagen synthesis. As such, vitamin C deficiency leads to serious complications, including vascular diseases. The aim of this study was to investigate the effects of vitamin C treatment on collar-induced intimal thickening. Rabbits were fed a normocholesterolemic diet and a non-occlusive silicon collar was placed around the left carotid artery for 3, 7, and 14 days. The rabbits were treated with or without vitamin C (150 mg/kg/day). Collar-induced intimal thickening became apparent at day 7. The effect of the collar on intimal thickening was more prominent at day 14. Vitamin C treatment significantly inhibited collar-induced intimal thickening at day 14. The placement of the collar around the carotid artery decreased maximum contractile responses against contractile agents (KCl, phenylephrine, 5-hydroxytryptamine). The effect of the collar on contractile responses was enhanced as days elapsed. Decreased contractile responses of collared carotid arteries normalized at day 14 in the vitamin C treatment group. Vitamin C treatment also restored sensitivity to phenylephrine. The collar also significantly decreased acetylcholine-induced relaxations at day 3 and day 7. Acetylcholine-induced relaxations normalized in collared-arteries in the placebo group at day 14. Vitamin C treatment significantly increased acetylcholine-induced relaxations of both normal and collared carotid arteries at day 14. MMP-9 expression increased in collared arteries at day 3 and day 7 but did not change at day 14. MMP-2 expression increased in collared arteries at day 14. However, vitamin C treatment reduced collar-stimulated expression of MMP-2 at day 14. These findings indicate that vitamin C may have potentially beneficial effects on the early stages of atherosclerosis. Furthermore these results, for the first time, may indicate that vitamin C can also normalize decreased contractile response through perivascular collar placement.

**Keywords:** vitamin C, collar, intimal thickening, rabbit carotid artery, vascular reactivity, MMP

## Introduction

The presence of thickened intima in atherosclerotic lesions has been recognized for nearly 150 years.<sup>1</sup> Intimal thickening is a physiological response to vascular damage and plays a critical role in development of atherosclerosis. The vascular damage that occurs in the vessel leads to endothelial dysfunction, vascular smooth muscle cell migration, and phenotype changes. In addition, intimal thickening is a complex process and many different cell types play a role in this process at different stages.<sup>1</sup>

Considering an abundance of experimental atherosclerosis models, various cellular mechanisms of intimal thickening were studied with the aim of enlightening the process and examining different treatment approaches. Placing a non-occlusive silicone collar around the carotid artery results in expedited intimal thickening. This model has therefore been used in many studies examining intimal thickening.<sup>2</sup> In this model, the changes at the intima layer are typically observed within 7 days and

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intimal thickening reaches a maximum on the 14th day.<sup>3,4</sup> Feeding experimental animals a hypercholesterolemic diet allows for the examination of different early stages of atherosclerosis.<sup>5,6</sup> Additionally, local application of various substances<sup>7,8</sup> and specific inhibitors or implementation of gene transfer<sup>9,10</sup> allows researchers to study the role of different mechanisms.

It is advantageous that the changes which occur in this experimental model are limited to the region where the collar is applied and that no visible damage occurs at the endothelium layer.<sup>11</sup> The geometric shape of applied collar is important in terms of the degree of intimal thickening.<sup>3,12</sup> Vascular reactivity changes have also been reported in many studies that were performed using the rabbit carotid artery silicone collar model.<sup>13–16</sup>

The various experimental and clinical studies on the effects of vitamin C on cardiovascular diseases are worth noting.<sup>17</sup> In many of these studies, vitamin C prevented endothelial dysfunction.<sup>18</sup> Specifically, vitamin C is effective against oxidative stress and inflammation in the cardiovascular system.<sup>19</sup>

In light of this information, the present research aimed to study the effects of vitamin C on intimal thickening, vascular responses, and morphometric parameters in the rabbit carotid artery collar model which is an effective experimental model in the research of intimal thickening.

## Materials and methods

### Materials

All agents were purchased from Sigma-Aldrich Co., St Louis, MO, USA. Primary and secondary antibodies were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Vitamin C was kindly provided by Roche, Turkey. All agonists used in organ bath experiments were dissolved in water and diluted in a 0.9% NaCl solution.

### Animals and treatment

Experimental protocol was approved by the ethics committee of the University of Ege, Faculty of Pharmacy. All animals received care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources.

Male New Zealand White rabbits (2–3 kg) were housed in individual cages and had ad libitum access to laboratory chow and water throughout the experiment period. The rabbits were divided into two groups: 150 mg/kg/day vitamin C (freshly

prepared each time) or the administration vehicle (distilled water) was administered by feeding tube (8 Ch, Bicakcilar, Turkey) to the rabbits.

### Collar-induced intimal thickening

Placement of the collar around the carotid artery has been previously described.<sup>15</sup> After 7 days of treatment, rabbits were anesthetized with sodium pentobarbitone, (30 mg/kg, intravenously). Both carotid arteries were surgically dissected from the surrounding tissues. A non-occlusive, flexible, biologically inert silicon collar was placed around the left carotid artery. The right carotid artery was sham-operated to expose a stretch similar to that exposed for the left carotid artery. The collar was left in position for 3 days, 7 days or 14 days. Then rabbits were killed with an overdose of sodium pentobarbitone and two segments (3 mm) were cut from left and right carotid arteries, one for immunohistochemistry and morphometric measurements and the other for organ bath studies. The first rings were immediately placed in a 4% buffered formalin solution for 24 hours and then embedded in paraffin blocks after routine processing.

### Organ bath studies

Two rings from each carotid artery were used. Rings were mounted between two stainless steel hooks in an organ chamber filled with 25 mL of 37°C Krebs solution continuously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The Krebs solution contained the following (in mM): NaCl, 118; KCl, 4.7; CaCl<sub>2</sub>, 2.5; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub>, 1.2; NaHCO<sub>3</sub>, 25 and glucose, 11.1. Contractile force changes were measured with an isometric force transducer (Grass FT03; Natus Neurology Incorporated, Middleton, WI, USA) and recorded by a computer program (IOSLab 3.23, France). The carotid artery rings were gradually stretched to a tension of 6 g. The rings were then allowed to equilibrate for 60 minutes at their optimal length. During the rest period, the Krebs solution in the organ chamber was changed every 15 minutes.

Carotid artery rings were contracted with a single dose of KCl (60 mM) at the end of the resting period. Next, concentration-response relationships to cumulative concentrations of phenylephrine (10<sup>-9</sup>–10<sup>-4</sup> M) were investigated in each ring. Cumulative concentration-relaxation curve to acetylcholine (10<sup>-9</sup>–10<sup>-4</sup> M) was constructed in the carotid artery rings followed by contraction with phenylephrine to 40%–60% of maximum phenylephrine contraction (submaximal contraction). Thereafter, concentration-response relationships to cumulative concentrations of serotonin (10<sup>-9</sup>–3×10<sup>-5</sup> M) and nitroglycerin (10<sup>-9</sup>–3×10<sup>-5</sup> M) were investigated.

## Immunohistochemistry and morphometry

Immunohistochemical staining was performed for Matrix metalloproteinase (MMP)-2 and -9 (neomarkers) using avidin-biotin peroxidase method as previously described.<sup>20</sup> Immunopositivity for MMP-2 and -9 was assessed using immunoscore by two pathologists who were blinded to the samples. The immunoscore procedures were performed semi-quantitatively. The staining area and staining intensity were graded on a scale from 0 to 4. The two scores were multiplied and the immunopositivity score was determined. The scores were then used to categorize immunopositivity as weak ( $\leq 2$ ) or strong ( $> 2$ ).

Transverse sections from each artery segment were cut and stained with Sirius red hematoxylin for morphometry. Images were captured at 4 $\times$  magnification using Nikon Eclipse 200 (Nikon Instruments, Melville, NY, USA). The cross-sectional area of intima, media, and adventitia was traced and measured by ImageJ. The area of lumen and external elastic lamina (EEL) was calculated as previously described.<sup>7</sup> The intima/media ratio (index) of each section was also calculated.

## Statistical analyses

All data were expressed as mean  $\pm$  standard error of the mean. Relaxation responses were expressed as percentage of phenylephrine pre-contraction. The negative logarithm of molar concentration of the agonist that produced a half-maximal contraction or relaxation for that agonist ( $pD_2$ ) was determined for each segment. For statistical analysis of organ bath and morphometry data, a factorial analysis of variance was used for treatment (vitamin C or placebo) between collar and sham-operated. Analysis was supplemented by an additional analysis (either a Wilcoxon signed ranked or Mann–Whitney *U*-test). A chi-square test was used to evaluate the statistical difference of immunostaining between the groups. Difference was considered significant at  $P \leq 0.05$ .

## Results

### Survival

All rabbits survived and tolerated the vitamin C treatment without any side effects. Neither collar placement nor vitamin C treatment changed the weight of the rabbits.

### Vascular morphometry

#### Luminal area

Placement of the collar around the carotid artery caused narrowing of the luminal area at day 3 and day 7. Collar-induced luminal area narrowing reached a maximum at day 14.

Vitamin C treatment did not affect this narrowing of the luminal area caused by the collar (Figure 1A). Despite the fact that the data for the luminal area of carotid arteries of vitamin C-treated rabbits seemed to indicate widening of the luminal area, the widening did not reach statistical significance (Figure 1A).

#### Tunica media

Placement of the collar caused significant reduction on the area of tunica media at day 3 and day 7 (Figure 1B). However, this effect of the collar on the area of tunica media disappeared at day 14. Vitamin C treatment did not affect the area of tunica media in both sham-operated and collared arteries (Figure 1B).

#### EEL

The area of EEL for each artery was calculated. Consistent with the results of tunica media, placement of the collar around the carotid artery decreased the area of EEL as compared to the sham-operated artery (Figure 1C). Vitamin C treatment did not affect the area of EEL. Neither vitamin C treatment nor placement of the collar changed the area of EEL (Figure 1C).

#### Tunica intima and index value

Collar-induced intimal thickening and increased index values were first significant at day 7 (Figure 2D, E). The effects of the collar on intimal thickening and index value were more prominent at day 14. Vitamin C treatment effectively reduced the intimal thickening and index value caused by the collar at day 14 (Figure 2D, E).

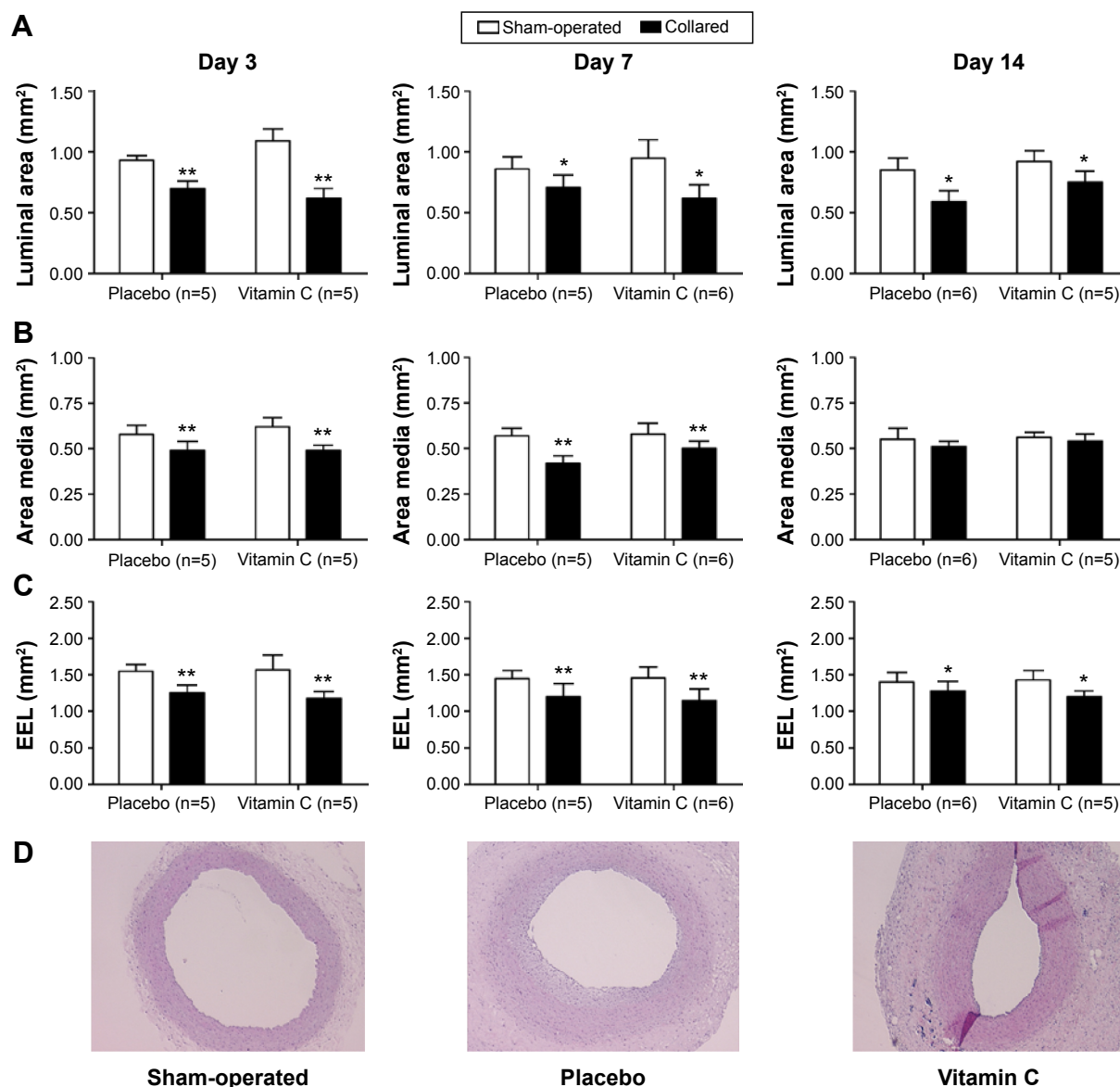
## Vascular reactivity

### Potassium chloride

Contractions to a single dose of KCl were significantly reduced in collared rings at all days. Vitamin C treatment normalized these reduced contractions at day 14 in collared rings. Vitamin C treatment did not affect contractions to KCl in sham-operated rings (Figure 3A).

### Phenylephrine

Placement of the collar around the carotid artery reduced contractions to phenylephrine. The inhibitory effect of the collar on phenylephrine contractions was maximized at day 14 (Figure 3B). Placement of the collar also caused decreased sensitivity to phenylephrine at all days (Table 1). Vitamin C treatment normalized reduced contractile response to phenylephrine at day 14 (Figure 3B). Vitamin C treatment



**Figure 1** Effects of collar and vitamin C on vascular morphometry.

**Notes:** Cross-sectional areas of lumen (A), media (B), and EEL (C). (D) Images of hematoxylin-eosin stained rabbit carotid arteries on day 14. Original magnification  $\times 4$ . Data are expressed as mean  $\pm$  SEM. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  sham-operated vs collared; Wilcoxon signed rank test.

**Abbreviations:** EEL, external elastic lamina; SEM, standard error of the mean.

also normalized sensitivity to phenylephrine at day 7 and day 14 (Table 1). Vitamin C treatment did not affect contractions to phenylephrine in sham-operated rings (Figure 3B).

### 5-hydroxytryptamine (5-HT)

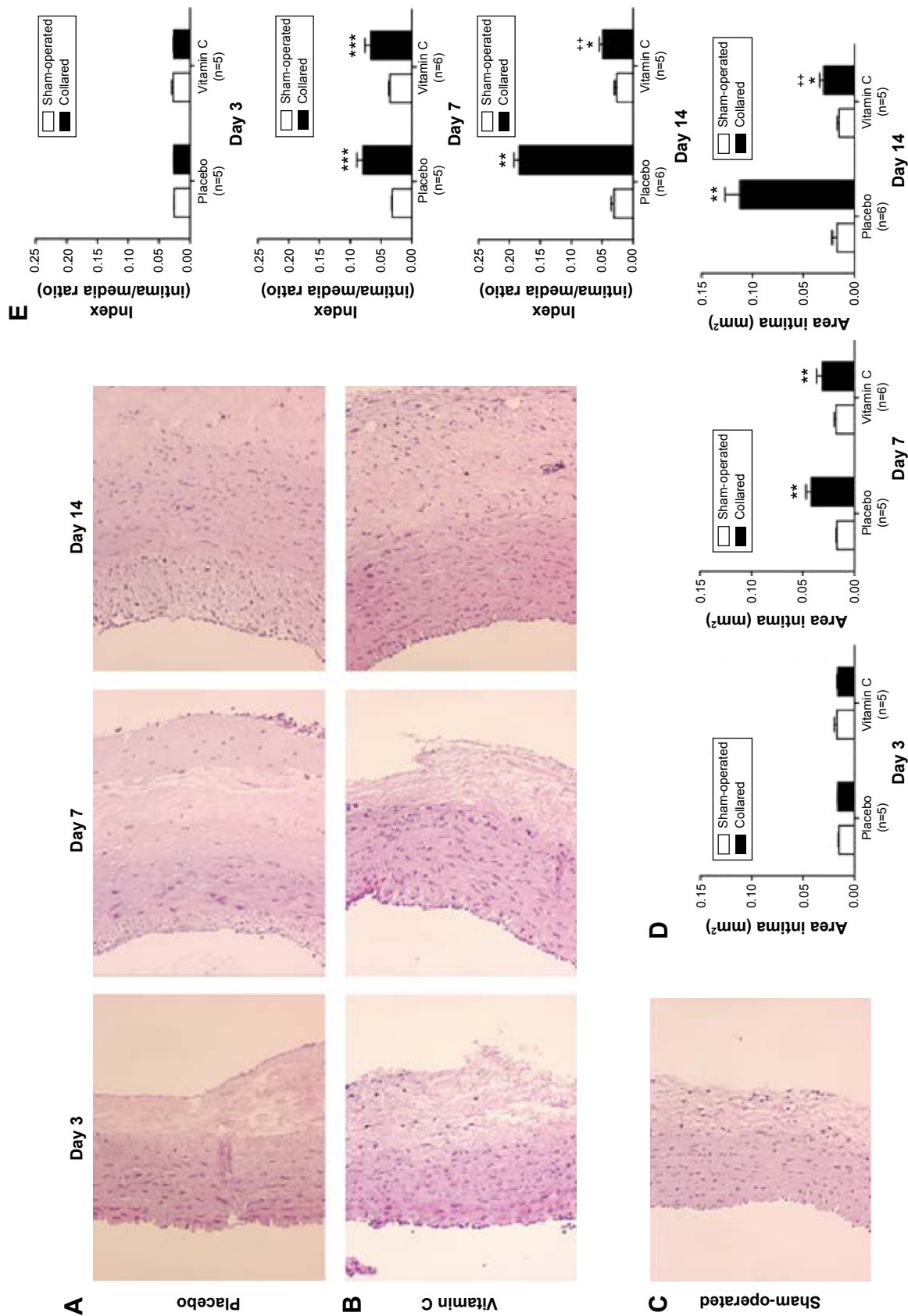
Similar to the responses to phenylephrine and KCl, placement of the collar around the carotid artery reduced contractile responses to 5-HT (Figure 3C) at all days. On the other hand, sensitivity to 5-HT was increased by the collar (Table 1). Vitamin C treatment normalized decreased contractile response to 5-HT (Figure 3C) at day 14. Regardless, vitamin C treatment did not normalize the increased

sensitivity to 5-HT (Table 1). Vitamin C treatment likewise did not affect contractions and sensitivity to 5-HT in sham-operated rings (Figure 3C).

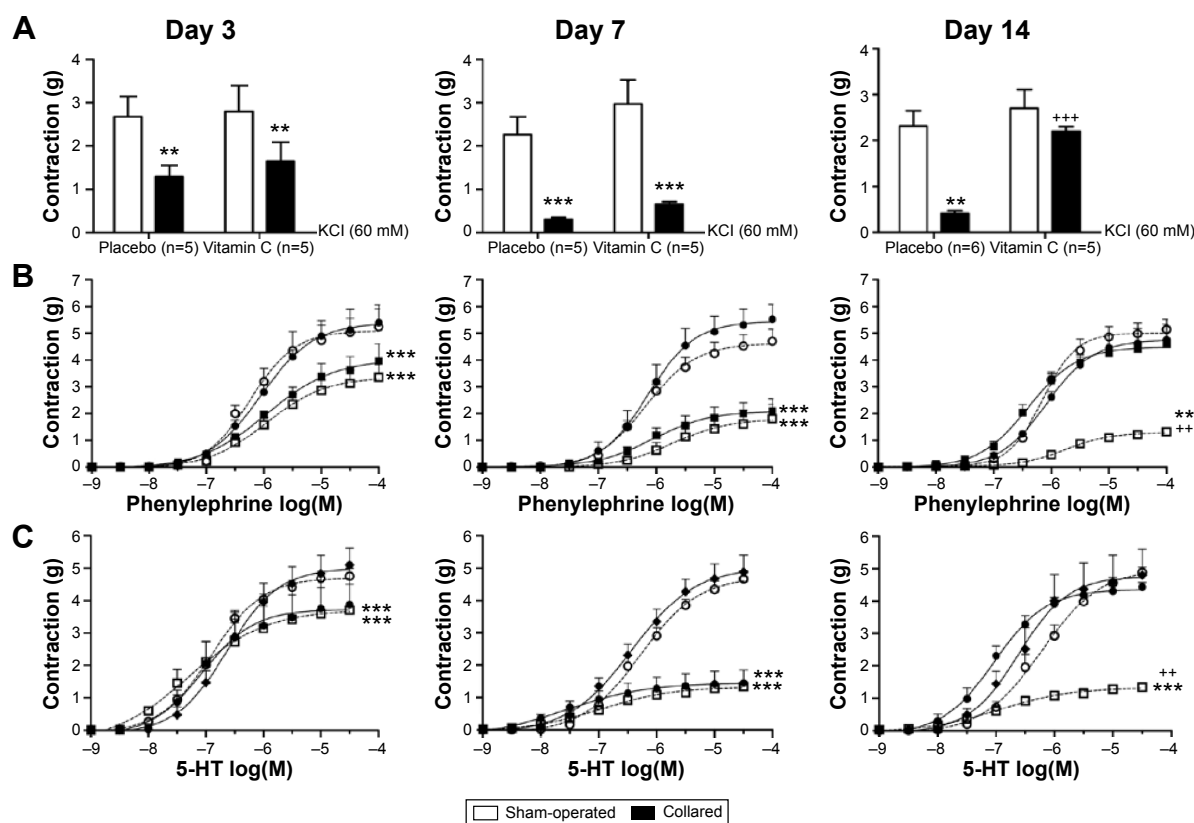
### Acetylcholine

Placement of the collar dramatically reduced the relaxation responses to acetylcholine as well as reduced sensitivity to acetylcholine on day 3 and day 7 (Figure 4A). However, placement of the collar did not affect relaxation responses to acetylcholine at day 14. Vitamin C treatment did not alter the effect of the collar on acetylcholine-induced relaxations at day 3 or day 7 (Figure 4A). Interestingly, vitamin C treatment





**Figure 2** Effects of collar and vitamin C on intimal thickening. **Notes:** (A–C) Images of hematoxylin-eosin stained rabbit carotid arteries. (A) Collared arteries from placebo group. (B) Sham-operated collared artery from placebo group (14th day). Original magnification  $\times 10$ . (D, E) Effects of collar and vitamin C on area intima (D) and index value (E). \* $P \leq 0.01$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  sham-operated vs collared; Wilcoxon matched signed-rank test. ++ $P \leq 0.01$  placebo vs vitamin C; Mann–Whitney U-test. Data are expressed as mean  $\pm$  SEM. **Abbreviation:** SEM, standard error of the mean.



**Figure 3** The effects of collar and vitamin C on the vascular contractions.

**Notes:** The effects of collar and vitamin C on the vascular contractions to KCl (**A**), phenylephrine (**B**), and serotonin (**C**). Cumulative dose-response curves (**B**, **C**) were made both in placebo-treated sham (○) and collar (□) rings and vitamin C-treated sham (●) and collar (■) rings. Data are expressed as mean ± SEM. \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  sham-operated vs collared; Wilcoxon matched signed-rank test. \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  placebo vs vitamin C; Mann-Whitney U-test.

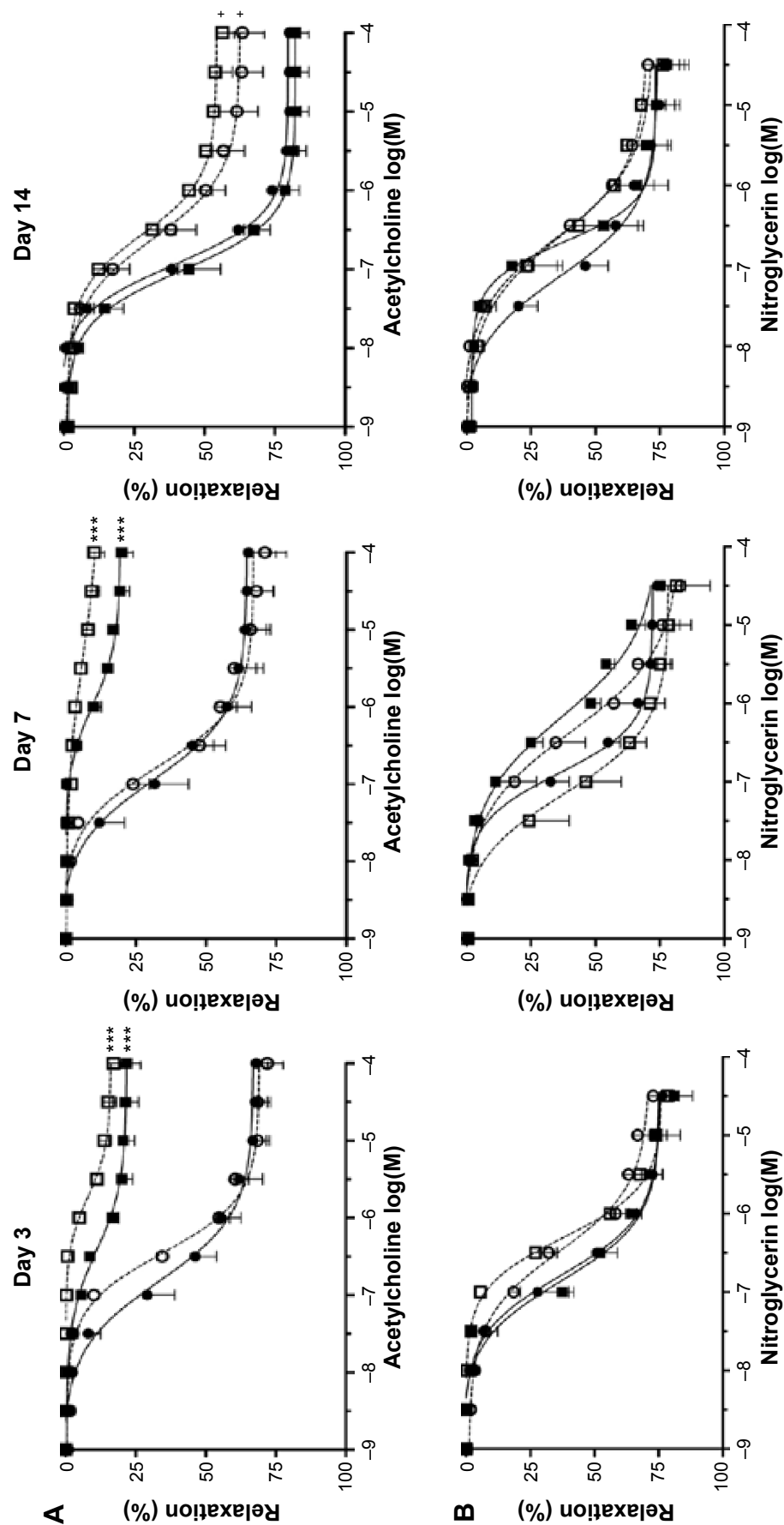
**Abbreviations:** SEM, standard error of the mean; 5-HT, 5-hydroxytryptamine.

**Table 1** Effects of collar and vitamin C on  $pD_2$  values to phenylephrine, 5-HT and acetylcholine

	Phenylephrine		5-HT		Acetylcholine	
	Placebo (n=5)	Vitamin C (n=5)	Placebo (n=5)	Vitamin C (n=5)	Placebo (n=5)	Vitamin C (n=5)
Day 3						
Sham	6.22±0.06	6.13±0.07	7.02±0.03	6.64±0.10	6.43±0.11	6.85±0.13
Collar	5.93±0.04	6.02±0.06	7.20±0.13	7.01±0.05	5.66±0.10*	6.62±0.22+
Significance of factors in analysis of variance						
– Collar	P=0.045		P=0.031		P=0.003	
– Vitamin C	NS (P=0.641)		P=0.022		P=0.010	
– Interaction	NS (P=0.223)		NS (P=0.440)		P=0.040	
Day 7						
Sham	6.15±0.11	6.13±0.12	6.29±0.11	6.42±0.09	6.74±0.03	6.87±0.14
Collar	5.47±0.17*	6.01±0.08+	6.99±0.18	7.33±0.10	6.12±0.34	5.98±0.24
Significance of factors in analysis of variance						
– Collar	P=0.007		P=0.001		P=0.018	
– Vitamin C	NS (P=0.105)		NS (P=0.102)		NS (P=0.784)	
– Interaction	P=0.042		NS (P=0.440)		NS (P=0.490)	
Day 14						
Sham	6.14±0.09	6.11±0.08	6.21±0.14	6.57±0.10	6.82±0.14	6.74±0.09
Collar	5.78±0.07*	6.40±0.09++	6.83±0.12	7.05±0.12	6.52±0.18	6.86±0.08
Significance of factors in analysis of variance						
– Collar	NS (P=0.452)		P=0.001		NS (P=0.448)	
– Vitamin C	P=0.021		NS (P=0.830)		NS (P=0.147)	
– Interaction	P=0.001		NS (P=0.516)		NS (P=0.991)	

**Notes:** Values are shown as mean ± SEM, n is the number of rabbits in each group. \* $P \leq 0.05$  sham-operated vs collared; Wilcoxon matched signed-rank test. \* $P \leq 0.05$ , \*\* $P \leq 0.01$  placebo vs vitamin C; Mann-Whitney U-test.

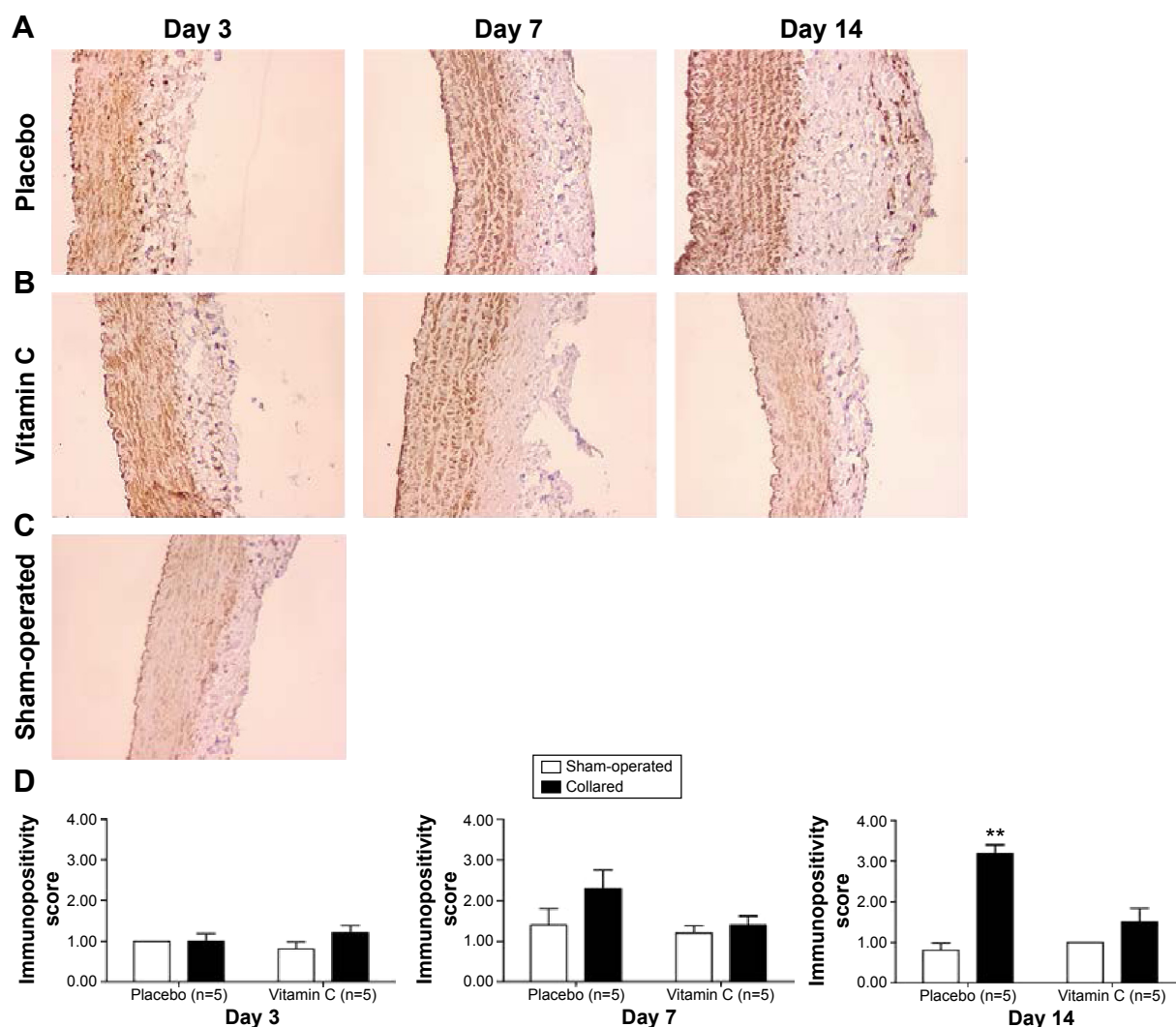
**Abbreviations:** 5-HT, 5-hydroxytryptamine; NS, not significant; SEM, standard error of the mean.



**Figure 4** The effects of collar and vitamin C on the vascular relaxations to acetylcholine and nitroglycerin.

**Notes:** Effects of collar and vitamin C on the vascular relaxations to acetylcholine (A) and nitroglycerin (B). Cumulative dose-response curves were made both in placebo-treated sham (○) and collar (□) rings and vitamin C-treated sham (●) and collar (■) rings. Data are expressed as mean ± SEM. \*\*\* $P \leq 0.001$  sham-operated vs collared; Wilcoxon matched signed-rank test. - $P \leq 0.05$  placebo vs vitamin C; Mann-Whitney U-test.

**Abbreviation:** SEM, standard error of the mean.



**Figure 5** Effects of collar and vitamin C on MMP-2 expression.

**Notes:** Images of MMP-2 immunohistochemically stained rabbit carotid arteries for MMP-2. **(A)** Collared arteries from placebo group. **(B)** Collared arteries from vitamin C group. **(C)** Sham-operated collared artery from placebo group (14th day). Original magnification  $\times 10$ . Data are expressed as mean  $\pm$  SEM. **\*\*** $P \leq 0.01$  sham-operated vs collared; chi-square test. **(D)** Immunopositivity scores of MMP-2 at day 3, 7 and 14.

**Abbreviation:** SEM, standard error of the mean.

increased acetylcholine-induced relaxations in both sham-operated and collared rings at day 14 (Figure 4A).

### Nitroglycerin

Neither placement of the collar nor vitamin C treatment affected relaxation responses to nitroglycerin in phenylephrine pre-contracted rings (Figure 4B).

## Immunohistochemistry

### MMP-2

Immunoscore data of MMP-2 showed that placement of the collar increased MMP-2 expression at day 14. Weak immunostainings of sham-operated arteries were limited at the medial area. Vitamin C treatment significantly inhibited increased MMP-2 expression (Figure 5).

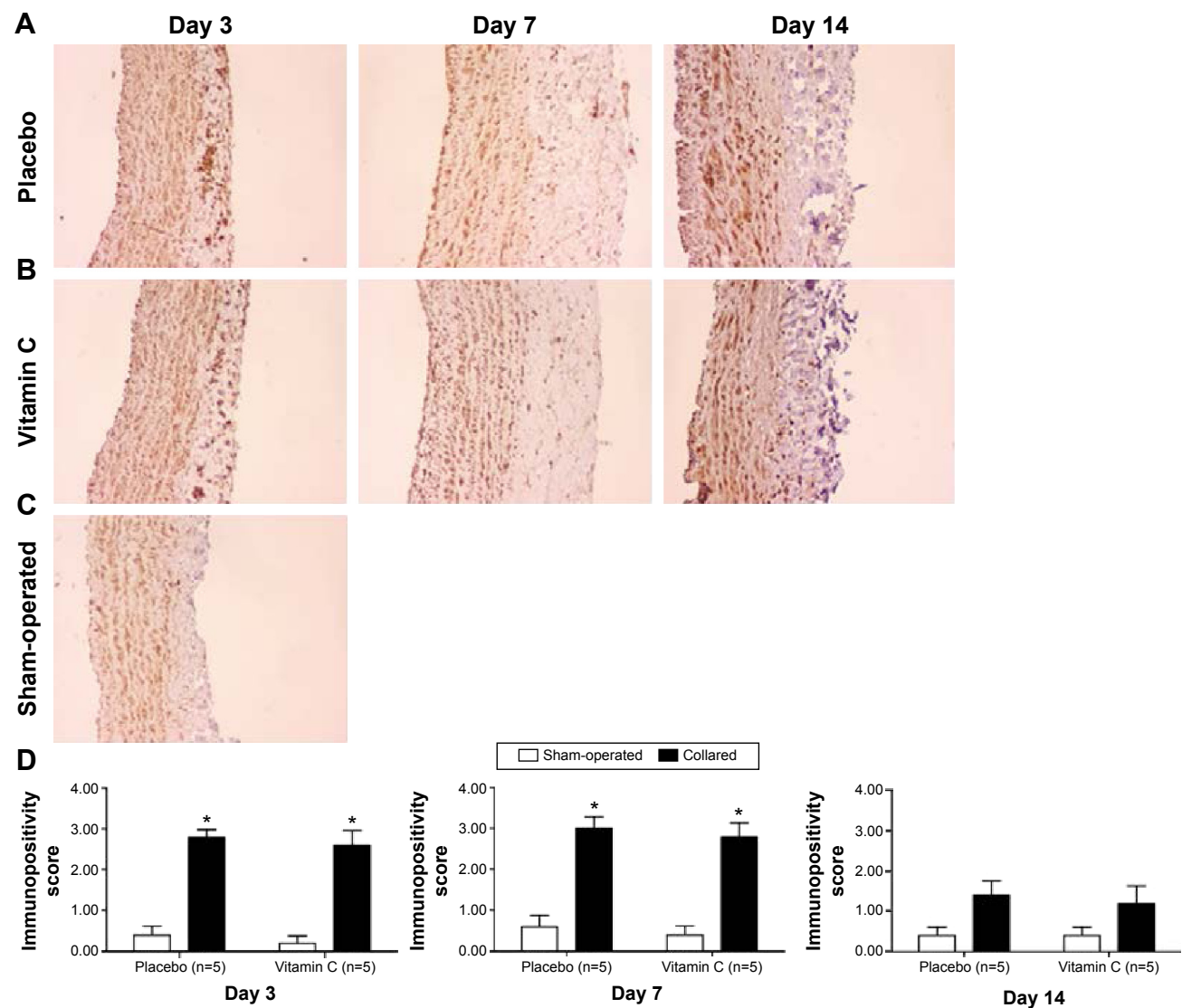
### MMP-9

Immunoscore data of MMP-9 showed that placement of the collar significantly increased MMP-9 expression at day 3 and day 7. Immunoscore data of MMP-9 did not show any significant difference between sham-operated and collared arteries at day 14, a finding in contrast to those previous days (Figure 6).

## Discussion

In the present study, the findings demonstrated that vitamin C treatment inhibited intimal thickening caused by placement of a collar around rabbit carotid artery and normalized decreased contractile responses at day 14. The results also showed that vitamin C treatment increased endothelium-dependent vasorelaxation in both collared and sham-operated arteries





**Figure 6** Effects of collar and vitamin C on MMP-9 expression.

**Notes:** Images of MMP-9 immunohistochemically stained rabbit carotid arteries for MMP-9. **(A)** Collared arteries from placebo group. **(B)** Collared arteries from vitamin C group. **(C)** Sham-operated collared artery from placebo group (14th day). Original magnification  $\times 10$ . Data are expressed as mean  $\pm$  SEM. \* $P \leq 0.05$  sham-operated vs collared; chi-square test. **(D)** Immunopositivity scores of MMP-9 at day 3, 7 and 14.

**Abbreviation:** SEM, standard error of the mean.

at day 14. Furthermore, vitamin C treatment also inhibited increased MMP-2 expression at day 14.

One of the most causative factors in luminal narrowing is intimal thickening. Placement of the collar caused luminal narrowing at all days in spite of there being no significant intimal thickening at day 3. Luminal narrowing at an early stage of intimal thickening caused by the placement of a collar was previously reported.<sup>21</sup> Yong et al reported that collaring caused decreased medial area, vasoconstriction and kinked carotid artery, in addition to changing blood flow at day 1 and day 7 of their study.<sup>21</sup> Similarly, the results of this study showed that collar placement resulted in decreased luminal area. Additionally, a reduction in medial area occurred at day 3 and day 7 in this study. Booth et al, who defined this

experimental intimal thickening model for the first time, noted in their study that collaring resulted in slight atrophy at medial layer at day 7.<sup>2</sup> In the current study, this finding gave rise to the concept that the combination of luminal area narrowing and decreases in EEL area with stenosis observed in the medial area at day 7 may be caused by intrinsic vascular remodeling. Vitamin C treatment did not demonstrate any effect on this vascular remodeling.

Intimal thickening that became apparent at day 7 and reached a maximum at day 14 was considerably inhibited by the treatment of vitamin C that continued for 21 days. The effect of vitamin C on intimal thickening did not seem robust on day 7. This may be because the dose of vitamin C was insufficient. Additionally, this phenomenon may indicate

that different subsequent mechanisms at different stages may play a role in the development of intimal thickening.

Beneficial effects of vitamin C on intimal thickening have been found in many clinical and experimental studies. One study reported that a combination of vitamin C and vitamin E decreased intimal thickening in pigs in which percutaneous transluminal coronary angioplasty (PTCA) was applied.<sup>22</sup> In a study that was performed on rats with periodontitis-induced atherosclerosis model, it was shown that vitamin C intake decreased the degree of atherosclerosis formed.<sup>23</sup> In another study of older males, it was shown that there was a reverse correlation between increased vitamin C intake and intimal thickness as assessed over 3 years.<sup>24</sup> A clinical study in which plasma levels of vitamin C and vitamin E were measured in patients with Alzheimer's disease, reported that tunica intima was thicker in the patients who had the lowest vitamin C plasma level.<sup>25</sup> A combination of vitamin C and vitamin E treatment also decreased intimal index in patients who had undergone cardiac transplantation in a separate study.<sup>26</sup>

In the rabbit carotid artery collar model, it was shown that placing of the collar resulted in the formation of reactive oxygen species (ROS) and upregulated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system that was a superoxide source at all layers of carotid artery.<sup>27,28</sup> Apocynin, an NADPH oxidase inhibitor, inhibited collar-induced intimal thickening by preventing an increase in superoxide.<sup>29</sup> When the role of superoxide in collar-induced intimal thickening is taken into consideration, it suggests that the strong antioxidant effect of vitamin C may have contributed to the inhibition of intimal thickening in this study.

Vascular adhesion molecules, growth factors, and chemokines may play an important role in the intimal thickening caused by collaring.<sup>9,28</sup> Vitamin C treatment may have beneficial effects on these molecules in the rabbit carotid artery collar model. Vitamin C can inhibit increased MCP-1 expressions.<sup>30,31</sup> Vitamin C treatment may also decrease expression levels of intercellular adhesion molecule (ICAM)-1 and vascular endothelial growth factor (VEGF).<sup>32,33</sup> Further studies that examine the relationship between these molecules and vitamin C will help define the underlying mechanisms of the effects of vitamin C on collar-induced intimal thickening.

The effects of both collaring and vitamin C on endothelium-dependent relaxations were examined in this study. It is known that the endothelium protects its physical integrity in the rabbit carotid artery collar model with the exception of some focal losses.<sup>11</sup> However, it was shown in this study that there was prominent endothelial dysfunction

at day 3 and day 7 following collar placement. This result is consistent with those of other studies in which acetylcholine-induced relaxations were examined.<sup>34,35</sup> As mentioned before, oxidative stress increases relatively quickly at an early stage after placement of the collar. The relationship between increased vascular oxidative stress and decreased nitric oxide vasodilation has been shown in many clinical and experimental studies.<sup>36</sup> This indicates that increased oxidative stress plays a significant role in collar-induced endothelial dysfunction. The absence of any difference among relaxation responses against nitroglycerine indicates that decreased acetylcholine response is not the result of a deterioration in the relaxation capacity of smooth muscle. However, these decreased acetylcholine induced relaxations were completely normalized at day 14. This finding is also consistent with other studies in which relaxation against acetylcholine was examined in a collar model.<sup>15,16,27,37,38</sup> Intimal iNOS and eNOS activity that became apparent 5 days after placement of the collar and adventitial iNOS activity that proved its presence at the 14th day supports the existence of a natural healing process in artery wall.<sup>39,40</sup> It was shown that vasodilation depending on endothelium was considerably maintained depending on effectivity of  $\text{Cu}^{+2}/\text{Zn}^{+2}$ -SOD despite the increase of superoxide production at the 14th day.<sup>27</sup>

In this study, the results indicated that vitamin C treatment increased acetylcholine-induced relaxations in both normal and collared carotid arteries. No observed increase at day 3 and day 7 may indicate that a definite period must expire for the vitamin C to improve endothelium-dependent relaxations. Vitamin C may improve endothelial functions via various mechanisms and increase endothelium-dependent relaxations.<sup>18</sup> In the studies performed on human endothelial cell culture and apo-E-deficient rats, vitamin C increased the synthesis of endothelial nitric oxide.<sup>41,42</sup> Vitamin C enhances nitric oxide synthase activity by increasing the chemical stabilization of tetrahydrobiopterin.<sup>41</sup> Vitamin C treatment for 8 weeks increased acetylcholine-induced relaxations in rat thoracic aorta.<sup>43</sup> It was reported in many clinical cases that vitamin C had beneficial effects on decreased endothelium-dependent relaxations in accordance with various conditions.<sup>44-47</sup>

It is known that MMPs and especially gelatinases play a critical role at the arterial wall in terms of intimal thickening.<sup>1</sup> In a study that was performed on ovariectomized rabbits fed a cholesterol-rich diet, collaring increased MMP-2, -3, and -9.<sup>5</sup> In a study in which the effects of TAK-044, endothelial receptor antagonist, on normocholesterolemic rabbits were examined, collaring increased MMP-2

immunoscores and resulted in no change in immunoscores of MMP-9 at day 14. However, the researchers also reported that there was an increase in active forms of MMP-2 and -9 in collared arteries.<sup>20</sup> Similar to previous studies, the collar led to an increase in MMP-2 immunoscores at day 14 in this study. However, it was observed for the first time in this study that there was an increase in MMP-9 immunoscores in collared arteries at day 3 and day 7 as well. Nitric oxide inhibition may lead to an increase in MMP-9 activity and expression and nitric oxide's existence may inhibit MMP-9 induction.<sup>48</sup> The increase in MMP-9 expression observed in collared arteries, in this study occurred in parallel to endothelial dysfunction. Therefore, decreased nitric oxide amount/activity may have played a role in the MMP-9 expression. Vitamin C treatment demonstrated no effect on MMP-9 expression. It did, however, prominently inhibit the increase in MMP-2 expression that we observed at day 14. Consistent with the findings from this study regarding the effects of vitamin C on MMP-2 expression, another study reported that vitamin C was effective in MMP-2, TIMP-1 and -2 expression in a rat abdominal aneurism model.<sup>49</sup> However, the findings from this study regarding gelatinases require further investigation, since the current study did not examine the protein expressions/mRNA levels of MMPs and activities of MMPs. Therefore, it cannot be determined whether vitamin C has an effect on the activities of gelatinases. Future studies that provide further insights on the expression levels, epigenetic regulation, and activities of gelatinases in different stages of collar-induced intimal thickening and how these factors interact with vitamin C treatment may increase the therapeutic value of vitamin C.

In this study, collar placement dramatically decreased contractions against all contractile agents. Reduction in contraction responses after collar placement is one of the most distinguishing characteristics of the collar model. This characteristic has been demonstrated among studies in which contraction responses were examined.<sup>14–16,38</sup> In all of these studies in which vascular reactivity was examined as well as intimal thickening, an improvement in decreased vascular contractions caused by the collar was not observed.<sup>15,16,38</sup> As such, it was previously thought that there was no correlation between intimal thickening and decreased contraction responses.<sup>16</sup> However, it was observed for the first time in this study that vitamin C nearly completely normalized decreased contraction responses as well as resulted in the inhibition of intimal thickening. These current findings draw attention to the importance of examining vascular contraction responses in intimal thickening studies. It is

widely known that vascular smooth muscle cells transform into synthetic phenotypes from contractile phenotypes in damaged vessels.<sup>50,51</sup> In their study, Reckless et al stated that there was a significant reduction in the levels of CD<sub>h</sub> isoform of caldesmon in collared arteries even at early periods when intimal thickening was hardly detected.<sup>51</sup> On the other hand, the levels of CD<sub>l</sub> isoform of caldesmon increased depending on the days.<sup>51</sup> It is remarkable that a reduction in CD<sub>h</sub> levels and an increase in CD<sub>l</sub> levels was observed parallel to decreased contraction responses depending on the day. Within this context, normalized contractile responses in collared arteries in the vitamin C treatment group may be related to the effect of vitamin C on the expression of contractile proteins specific to vascular smooth muscle. Interestingly, a previous study demonstrated that vitamin C increased expressions of h1-calponin, SM22 $\alpha$ , and  $\alpha$ -actin in vascular smooth muscle cells.<sup>52</sup> Accordingly, a subsequent in vivo study also reported that vitamin C stimulates expression of genes specific to smooth muscle cells in smooth muscle cells and prevents synthetic type differentiation of smooth muscle cells.<sup>53</sup> Further investigation will broaden the researchers' knowledge of the expressions of contractile proteins and phenotypic changes of vascular smooth muscle regarding effects of vitamin C on intimal thickening. Additionally, increased MMP-2/-9 activity and/or expression may have a role in decreased contractions in collar-induced intimal thickening. In a study of rat aorta, MMP-2 and -9 significantly inhibited phenylephrine-induced contractions.<sup>54</sup> Therefore, the effects of vitamin C on MMP-2 expression may contribute to its beneficial effects on decreased contractions caused by the collar.

Taken together, the findings from this study may provide new insights into the effects of vitamin C on intimal thickening. Furthermore, to the best of the researchers' knowledge, this study marks the first time it has been shown that vitamin C inhibited intimal thickening and enhanced decreased contractile responses in collar-induced intimal thickening. In terms of applicability, this study adds valuable knowledge regarding the clinical value of vitamin C and may serve to differentiate vitamin C from other antioxidants and their implications in cardiovascular disease.

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## Disclosure

The authors have no competing interests to declare.

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